

Acids and Esters of Flue-Cured Tobacco

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ABSTRACT

The isolation and quantitative determination of certain acids and esters from tobacco are described. Based on infrared spectrophotometric and gas chromatographic findings, the compounds were tentatively identified as free higher fatty acids and methyl and ethyl esters thereof.

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EASTERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION

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ACIDS AND ESTERS OF FLUE-CURED TOBACCO

by

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Numerous workers have reported the presence of free and esterified higher fatty acids in tobacco leaf (9, 19, 20, 7, 11, 12, 13, 14, 5, 18)*, in tobacco seed (16, 10, 23, 15), and in tobacco smoke condensate (1, 24, 3, 22, 17). Qualitatively, the free acids, lauric, myristic, palmitic, oleic, linoleic, and linolenic, have been isolated from leaf. The presence of C_{12} - C_{20} saturated acids in the free state has also been shown by Hellier (5) using paper chromatographic procedures. Quantitatively, Hellier has estimated the amounts of total free and combined saturated and unsaturated fatty acids, and Carruthers and Johnstone (2) have shown the proportions of fatty acids in cigarette tobacco using gas chromatography and mass spectrometry. The chief component of the free-acid mixture is believed to be palmitic acid.

In the case of smoke, the work of Clemo (3) has shown the presence and quantities of at least 15 free fatty acids in the C_{14} - C_{26} range in cigarette smoke condensates. Of these, 9 were identified as acids commonly encountered in natural lipoid mixtures. The chief free-fatty acid components were palmitic, linoleic, and linolenic acids.

With respect to combined higher fatty acids, the presence of esters of sterols, solanesol, and glycerol in smoke or leaf has been shown and the occurrence of many others, for example, esters of carotenoids, suspected. Among the esters derived from lower aliphatic alcohols, Izawa and Kobashi (8) have isolated the ethyl esters of steam-volatile fatty

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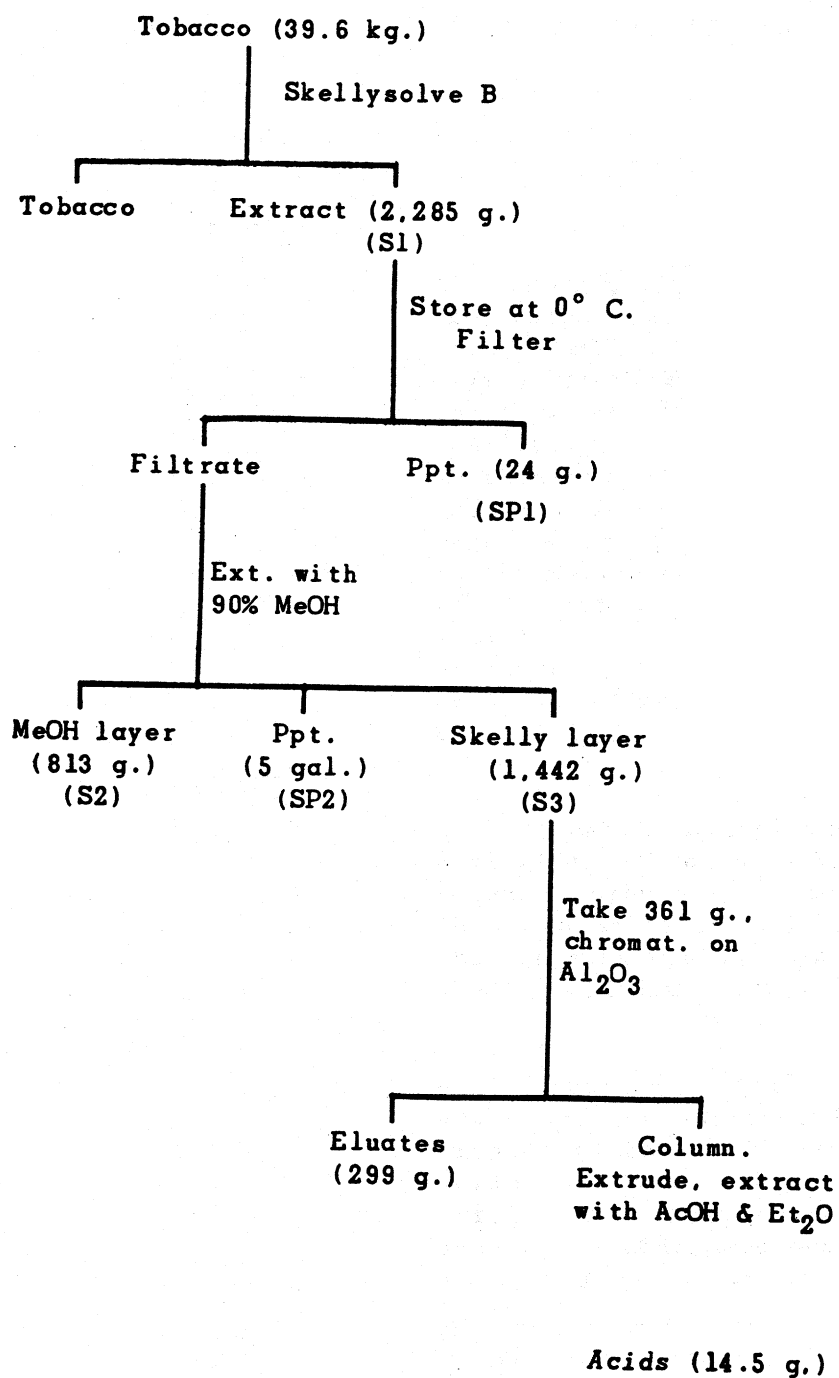


Fig. 1. Isolation of the acids from flue-cured tobacco

acids from cigarette smoke condensates and Onishi, et al. (11) have reported the occurrence of methyl laurate, methyl myristate, and methyl palmitate in the leaf.

The present report describes a cursory survey of certain acids and esters obtained from the Skellysolve-soluble and ethanol-soluble materials extractable from flue-cured leaves. On the basis of infrared spectrophotometric and gas chromatographic findings, the compounds have been tentatively identified as free and esterified fatty acids. Data on the amounts of such acids and esters in Type 12 leaves are also given.

METHODS, RESULTS, AND DISCUSSION

Acids

These compounds were obtained from the large-scale extraction of tobacco, which was previously described (4). Unaged Type 12 leaf webs (39.6 kilograms) were extracted with Skellysolve B*, which removed 2,285 grams of solids (see Figure 1). The Skellysolve extract (10.8 liters) (S1) was stored at 0°C. for 8 days, removed, and filtered, giving 24 grams of solid. The filtrate was then successively extracted with three portions (6 liters each) of 90 percent methanol and three portions (2 liters each) of water. A precipitate (5 grams) formed at the methanol-Skellysolve interface and this was collected. The methanol extracts were combined and found to contain 813 grams of solids. The Skellysolve layer was then dried over magnesium sulfate and one-fourth of the total extract (361 grams) was chromatographed on a column (3 inches by 37 inches) containing 9.2 pounds of activated acid-washed alumina. The column was developed by a flowing technique beginning with petroleum ether and ending with water. After this elution approximately 62 grams of material still remained on the column.

* MENTION OF A SPECIFIC COMMERCIAL PRODUCT DOES NOT CONSTITUTE ENDORSEMENT BY THE UNITED STATES DEPARTMENT OF AGRICULTURE.

The column was extruded and the alumina was suspended in 1.5 liters glacial acetic acid. The mixture was placed on the steam bath and agitated for 45 minutes, during which time the temperature of the mixture was 70° to 80° C. The acetic acid was decanted off, another 1.5 liters glacial acetic acid was added, the hot extraction was repeated, and the mixture was permitted to cool and remain at room temperature overnight. The acetic acid was then filtered off, the alumina suspended in 2 liters diethyl ether, and the mixture was stirred and filtered. A third extraction of the alumina with acetic acid followed by diethyl ether was performed in the same manner.

To each of the acetic acid extracts were added two parts of water, and the mixtures were refrigerated for 3 to 24 hours. The resulting precipitates were filtered off and dissolved in diethyl ether. These ethereal solutions and the above ethereal extracts were washed thoroughly with water and dried over magnesium sulfate. A total of 14.5 grams of material was eluted from the alumina by this procedure. Undoubtedly, more material remained on the alumina; however, further extraction was not performed since the alumina has become gelatinous and difficult to handle.

Each of the ethereal solutions was evaporated to an oily or waxy residue, and each of the residues was crystallized from hot methanol. Multiple recrystallizations of certain of these fractions were attempted and several solids (total weight, 3.5 grams) melting within a 1° to 2° range from 50° to 70° C. were obtained. The filtrates from all crystallizations were combined and the solvent evaporated to give a thick oily residue. The residue (11 grams) was distilled at 0.5 millimeter Hg. and three fractions (total, 5.5 grams) boiling in the 157° to 191° C. range were collected. The nonvolatile matter in the still pot was taken up in ether and crystallized from methanol after evaporation of the solvent. The filtrate from the crystallization was evaporated to a dark, tacky mass (A, 4.0 grams).

The infrared absorption spectra of certain of the crystallized and distilled fractions were determined

and all samples showed the characteristics of long-chain fatty acids. Each fraction was then methylated, using diazomethane, and the resulting methyl esters examined by vapor phase chromatography. Each fraction was run under at least two different conditions of column temperature and helium flow rate (see Table 1). With the exception of Fraction A, which was not volatile under the conditions employed, all fractions gave elution curves essentially comparable to an authentic mixture of methyl esters of higher fatty acids. Although the various fractions differed qualitatively and quantitatively, all were found to contain the commonly occurring higher fatty acids as major constituents. This would indicate that the elution of the acids from the alumina was relatively nonselective. At least 16 compounds were detected in the various mixtures; of these, 7 could be tentatively identified by comparison with reference compounds, and 2 could be estimated (Table 1). The remaining 7 were

Table 1. -- Percentage composition of the acids from cured Type 12 tobacco*

Peak	Percentage	Peak	Percentage
A	trace	Stearic	
B	trace	and oleic	18
C	trace	Linoleic	15
Capric (?)	<1	Linolenic	30
Lauric	<1	E	<1
Myristic	2	F	1
D	4	G	<1
Palmitic	26	H	<1
Palmitoleic (?)	2		

* DETERMINED BY GAS CHROMATOGRAPHIC ANALYSIS OF THE METHYL ESTERS USING AN AEROGRAPH A-100 INSTRUMENT, A 5 FEET BUTANDIOL SUCCINATE POLYESTER COLUMN AND A NUMBER OF EXPERIMENTAL CONDITIONS. STEARATE AND OLEATE COULD NOT BE CLEARLY SEPARATED FOR QUANTITATIVE DETERMINATION.

unidentified. An overall percentage of each component in the entire series of fractions was then determined. These values are given in Table 1 and should be considered as estimates rather than rigid analytical values.

The data indicate that palmitic and linolenic acids are the main higher fatty acid components with the latter compound being slightly greater. Common unsaturated acids represent almost one-half of the total acids present. In general, the trend of these data is in agreement with the findings of Clemo (3) on the quantitative pattern of higher fatty acids in cigarette smoke condensate and the data of Carruthers and Johnstone (2).

Based on the actual weight of recovered fatty acids extracted from the alumina, the minimum total concentration of these compounds in tobacco is 0.11 percent. The maximum level of acids extractable with Skellysolve B and insoluble in aqueous methanol would be 0.65 percent, assuming that 70 percent* of all substances remaining on the alumina after the completion of the elution scheme were free fatty acids. It should be emphasized that the present work concerns only the higher fatty acids extractable with Skellysolve which did not partition into 90 percent methanol.

In connection with the fatty-acid composition of tobacco, it is of interest to note that the higher fatty acids are believed to influence the mildness of tobacco smoke (13).

Esters

These compounds were obtained from fraction EP3, which was previously described (4). Briefly, the origin of this fraction was as follows: After extraction of unaged, cured Type 12 tobacco with Skellysolve B, as outlined above, the tobacco was exhaustively extracted with ethanol. On concentration of the

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alcoholic extract and storage at 0° C., a precipitate was obtained. The neutral substances (Figure 2, EP3, 140 grams) in the water-insoluble material from this precipitate were chromatographed on acid-washed alumina. The column was developed with a succession of solvents and campesterol was obtained from the fractions eluted with 1:1 benzene-diethyl ether, as previously described. The esters were then isolated from the material eluted with petroleum ether (EP4, 99 grams).

Thirty grams of fraction EP4 were chromatographed on a column (1.5 inches by 16 inches) of acid-washed alumina. Elution was performed with a number of solvents and several fractions collected. The sol-

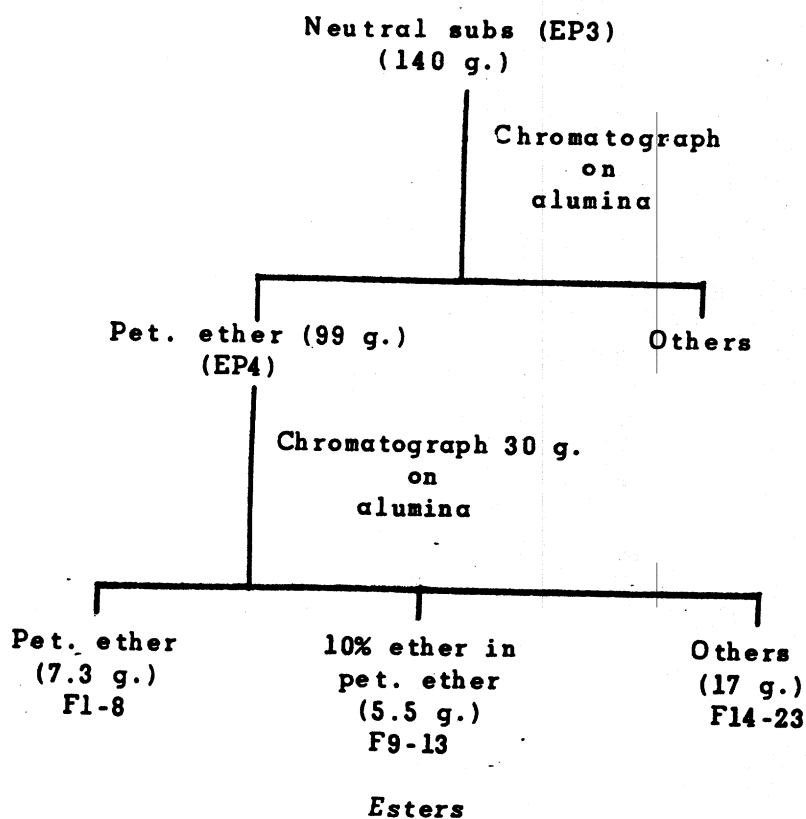


Fig. 2. Isolation of the esters from cured Type 12 tobacco leaves.

Table 2. -- Percentage composition of the esters
from cured Type 12 tobacco.*

Peak	Fraction Number				
	9	10	11	12	13
	(Percentage)				
A	<1	<1	1	5	5
B	<1	<1	1	3	5
C	-	<1	-	-	3
Lauric	1	<1	2	-	4
C ₁	<1	<1	<1	-	-
C ₂	<1	-	<1	-	-
Myristic	3	2	1	4	3
D	<1	3	1	-	4
D ₁	<1	-	3	-	-
Palmitic	35	38	17	9	4
Palmitoleic (?)	<1	-	-	-	7
Stearic-oleic	20	12	18	8	3
Linoleic	21	21	24	14	3
Linolenic	18	24	31	57	54
F	-	-	-	-	4

* DETERMINED BY GAS CHROMATOGRAPHIC ANALYSIS USING AN AEROGRAPH A-100 INSTRUMENT, A 5 FEET BUTANDIOL SUCCINATE POLYESTER COLUMN AND A NUMBER OF EXPERIMENTAL CONDITIONS.

vents, number of fractions, and total weights of fractions were as follows: Petroleum ether (1.3 liters, 8 fractions, 7.3 grams), 10 percent diethyl ether in petroleum ether (0.45 liter, 5 fractions, 5.5 grams), 20 percent ether in petroleum ether (0.5 liter, 2 fractions, 1.0 gram), 30 percent ether in petroleum ether (0.6 liter, 2 fractions, 1.5 grams), 1:1 ether-petroleum ether (1.2 liters, 3 fractions, 2.5 grams), and ether (1.3 liters, 3 fractions, 12 grams).

The infrared absorption spectra of the five fractions eluted with 10 percent ether in petroleum ether were very similar and showed the characteristics of methyl esters of the higher fatty acids. Accordingly, each fraction was analyzed directly by the vapor phase chromatographic methods described above. A number of peaks were obtained from each of the fractions and certain of these peaks showed elution times comparable to those of authentic methyl esters of palmitic, stearic, oleic, linoleic, and linolenic acids. The percentage composition of each fraction was estimated, and the results are given in Table 2. Fifteen compounds were detected in the five samples. In general, the predominating components were esters of palmitic and higher unsaturated acids.

Fractions 15, 18, and 20, eluted with increasing concentrations of diethyl ether in petroleum ether, were also examined by vapor phase chromatography although the infrared spectra of these suggested a predominance of lower terpenes instead of fatty-acid esters. The elution curves of fractions 15 and 18 showed the presence of some esters of fatty acids; fraction 20 gave no peaks indicative of these esters but showed gradual elution in regions in which geraniol, menthol, and camphor are eluted.

Experiments with authentic methyl and ethyl linoleate indicated that no separation of these esters could be achieved under the gas chromatographic conditions used. Likewise, differentiation of methyl and ethyl esters was not possible on the basis of infrared spectra. Thus, even tentative identification of the methyl esters of the fatty acids could not be estab-

lished from the gas chromatographic or infrared spectral data. Accordingly, fractions 12 and 13 were combined and saponified in diethylene glycol containing 3.5 percent potassium hydroxide. The saponification was conducted in a system having a side trap filled with an ethereal solution of phenyl isocyanate for the collection of volatile constituents. After hydrolyzing the sample at 125° C. for 1 hour, the side trap was removed and the contents were placed in an evaporating dish at room temperature overnight. The residue thus obtained was recrystallized three times from diethyl ether giving white crystals, m.p. 48.5° to 50.5° C. Authentic samples of methyl phenylurethan, m.p. 47.5° to 47.9° C., and ethyl phenylurethan, m.p. 49.5° to 50.5° C., were prepared and a mixed melting point determination* of each with the above crystals was performed. The unknown derivative gave no depressions with authentic ethyl phenylurethan (m.p. mixture, 48.5° to 50.5° C.); on mixing the unknown with the methyl compound, the mixture softened at room temperature and melted at 29° to 46° C. Experiments on various mixtures of authentic methyl and ethyl phenylurethans showed that combinations containing as small as a 5 percent methyl compound depressed the melting point of the ethyl derivative significantly (softening at 42° C., melting at 44° to 49° C.).

Fractions 9-11 were then combined and saponified in diethylene glycol containing 1.0 N potassium hydroxide. An ordinary distillation setup was employed with a receiver cooled in dry ice so that all volatile constituents were trapped during the course of the saponification. The bath temperature was raised from 115° C. to 156° C. over a 1-hour period and then held at 156° to 157° C. for 20 minutes. The condenser was rinsed with a small amount of petroleum ether that was collected in the receiver. The combined distillate and rinsings were then analysed by gas chromatography. The results are given in Table 3.

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Table 3. -- Gas chromatographic separation of the alcoholic components of the esters from tobacco*

Sample	Column temp.	Carrier gas rate	Retention time
	(°C.)	(ml. He./min)	(min)
Authentic:			
Methanol	100	28	4.2
Ethanol	100	28	4.9
Methanol	101	55	2.0
Ethanol	101	55	2.2
Methanol	119	57	1.4
Ethanol	119	57	1.5
Unknown:			
Peak #1	100	28	4.2
Peak #2	100	28	4.9
Peak #1	101	55	2.0
Peak #2	101	55	2.2
Peak #1	119	57	1.3
Peak #2	119	57	1.5

AEROGRAPH MODEL A-100 INSTRUMENT, 5 FEET COLUMN CARBOWAX, 250 MILLI-AMPERES FILAMENT CURRENT AND 40 INCHES PER HOUR RECORDER SPEED. AUTHENTIC ALCOHOLS INJECTED IN SMALL AMOUNTS (5 MICROLITERS) TO SIMULATE PEAK SIZE OBTAINED WITH UNKNOWN PEAKS.

The distillate showed two main peaks, which, under three sets of chromatographic conditions, had retention times similar to ethanol and methanol. Area measurements indicated that the ratio of the peaks corresponding to ethanol and methanol was approximately 2:1. A small peak giving the same retention time as water was also observed in the distillate. Although normal alcohols up to C₇ could be eluted at 119° C. and 57 milliliters helium per minute, no peaks having retention times greater than ethanol with the exception of water were found in the distillate.

It should be noted that the gas chromatographic demonstration of both methanol and ethanol in the saponification mixture of fractions 9-11 is at variance with the above data showing only ethanol in the saponification mixture of fractions 12-13. Perhaps the latter fractions contained only ethyl esters, whereas fractions 9-11 were mixtures of methyl and ethyl compounds. Possibly, the recrystallizations used to purify the unknown phenylurethan served as a fractional crystallization, separating the methyl from the ethyl derivatives. At any rate, on the basis of the gas chromatographic findings, infrared spectral characteristics and the isolation of ethyl phenylurethan, it was tentatively concluded that the compounds isolated from tobacco were mixtures of methyl and ethyl esters of higher fatty acids.

Since these esters were obtained from an ethanolic extract of leaves, the possibility that the ethyl compounds are artifacts should be mentioned. Conceivably, esterification of the free-fatty acids by the extracting solvent could have occurred. This question cannot be conclusively decided in the present work. However, at least two points should be mentioned. The quantitative aspects of such an esterification would appear to be quite fortuitous, since the levels of ethyl and methyl compounds are within the same general range of concentration. Also, evidence has been presented by others (11, 8) showing the presence of ethyl esters of fatty acids in tobacco leaves under conditions in which artifacts could not have contributed to the findings.

SUMMARY

Mixtures of acids having the infrared spectral characteristics of higher fatty acids were isolated from a Skellysolve B extract of cured Type 12 tobacco. Vapor phase chromatographic studies of the methyl esters derived from these acids showed the presence of at least 16 compounds of which 7 gave retention times identical with those of authentic methyl esters of lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic acids. The percentage composition of the acids indicated that linolenic and palmitic acids were the main components.

From an ethanolic extract of the above tobacco were isolated several mixtures of esters. Vapor phase chromatographic and infrared spectral characteristics indicated the presence of methyl and ethyl esters of higher fatty acids therein; the possibility that the ethyl esters are artifacts was discussed. Percentage composition studies indicated a predominance of esterified palmitic and higher unsaturated acids in the mixtures.

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